Binding of DEAD-box helicase Dhh1 to the 5′-untranslated region of *ASH1* mRNA represses localized translation of *ASH1* in yeast cells

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Local translation of specific mRNAs is regulated by dynamic changes in their subcellular localization, and these changes are due to complex mechanisms controlling cytoplasmic mRNA transport. The budding yeast *Saccharomyces cerevisiae* is well suited to studying these mechanisms because many of its transcripts are transported from the mother cell to the budding daughter cell. Here, we investigated the translational control of *ASH1* mRNA after transport and localization. We show that although *ASH1* transcripts were translated after they reached the bud tip, some mRNAs were bound by the RNA-binding protein Puf6 and were non-polysomal. We also found that the DEAD-box helicase Dhh1 complexed with the untranslated *ASH1* mRNA and Puf6. Loss of Dhh1 affected local translation of *ASH1* mRNA and resulted in delocalization of *ASH1* transcript in the bud. Forcibly shifting the non-polysomal *ASH1* mRNA into polysomes was associated with Dhh1 dissociation. We further demonstrated that Dhh1 is not recruited to *ASH1* mRNA co-transcriptionally, suggesting that it could bind to *ASH1* mRNA within the cytoplasm. Of note, Dhh1 bound to the 5′-UTR of *ASH1* mRNA and inhibited its translation *in vitro*. These results suggest that after localization to the bud tip, a portion of the localized *ASH1* mRNA becomes translationally inactive because of binding of Dhh1 and Puf6 to the 5′- and 3′-UTRs of *ASH1* mRNA.

Local translation of particular mRNAs involves dynamic changes in subcellular localization. To this end, cells employ various and complex mechanisms to control specific cytoplasmic localization of mRNAs and their site of translation (1). The budding yeast *Saccharomyces cerevisiae* has emerged as a good system to study these mechanisms because a substantial number of transcripts have been identified as transporting from the mother cell to the budding daughter cell for such a purpose (2). One of these transcripts, *ASH1*, is transcribed in the mother cell and then transported to the bud tip, where the protein is translated and translocated into the daughter cell nuclei during late anaphase (3). The asymmetric localization of Ash1 within the daughter cell nuclei represses the transcription of HO endonuclease, which is crucial for the mating-type switch (3–5). The localization of *ASH1* mRNA relies on She2, an RNA-binding protein that is co-transcriptionally bound to the cis-acting elements (or "zipcodes") of the mRNA (6, 7). After transcription, the *ASH1* mRNP[2] complex is exported to the cytoplasm, where it interacts with She3, an adaptor protein for the mRNA localization machinery (8). She3 connects the *ASH1* mRNP complex to the motor protein She1 (Myo4), which utilizes the actin cytoskeleton to transport the complex to the bud tip (9).

In addition to She2, other RNA-binding proteins, such as Puf6 and Khd1, have been identified as essential for complete localization of *ASH1* mRNP complex and subsequent local translation of Ash1. Khd1p was identified in a systematic survey of potential candidate RNA-binding proteins for *ASH1* mRNA localization (10). Binding of Khd1 decreased translational initiation of *ASH1* mRNA and reduced leakage of Ash1 into the mother cell nucleus (11). Puf6 is a member of the Pumilio/FBF (*fem-3* mRNA-binding factor) family of RNA-binding proteins that was purified from She2-associated mRNPs (12). Puf6 directly binds to the 3′-UTR of *ASH1* mRNA and represses its translation during the transport process. At the bud tip, phosphorylation of Puf6 releases translational repression of *ASH1* mRNA and leads to Ash1 synthesis (13). These studies indicate that translation of *ASH1* mRNA is precisely regulated by RNA-binding proteins, and Puf6 and Khd1 could function in the linkage between *ASH1* mRNA localization and its translation. Although translational repression of *ASH1* mRNA during transport has been well studied, the regulatory mechanism for *ASH1* mRNA translation after its proper localization remains to be studied.

In this study, we show that although translation of *ASH1* mRNA occurs after localization, a portion of the transcripts remain non-polysomal and therefore could still bind to Puf6. We identified Dhh1, which interacts with untranslated *ASH1* mRNA and co-precipitates with Puf6. Deletion of the *DHH1* gene not only results in diffusion of *ASH1* mRNA in the bud but also

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This article contains supplemental Figs. S1–S6.

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[2] The abbreviations used are: mRNP, messenger ribonucleoprotein; MBP, maltose-binding protein; MCP, MS2-binding coat protein; qPCR, quantitative PCR; CHX, cycloheximide; IP, immunoprecipitation.

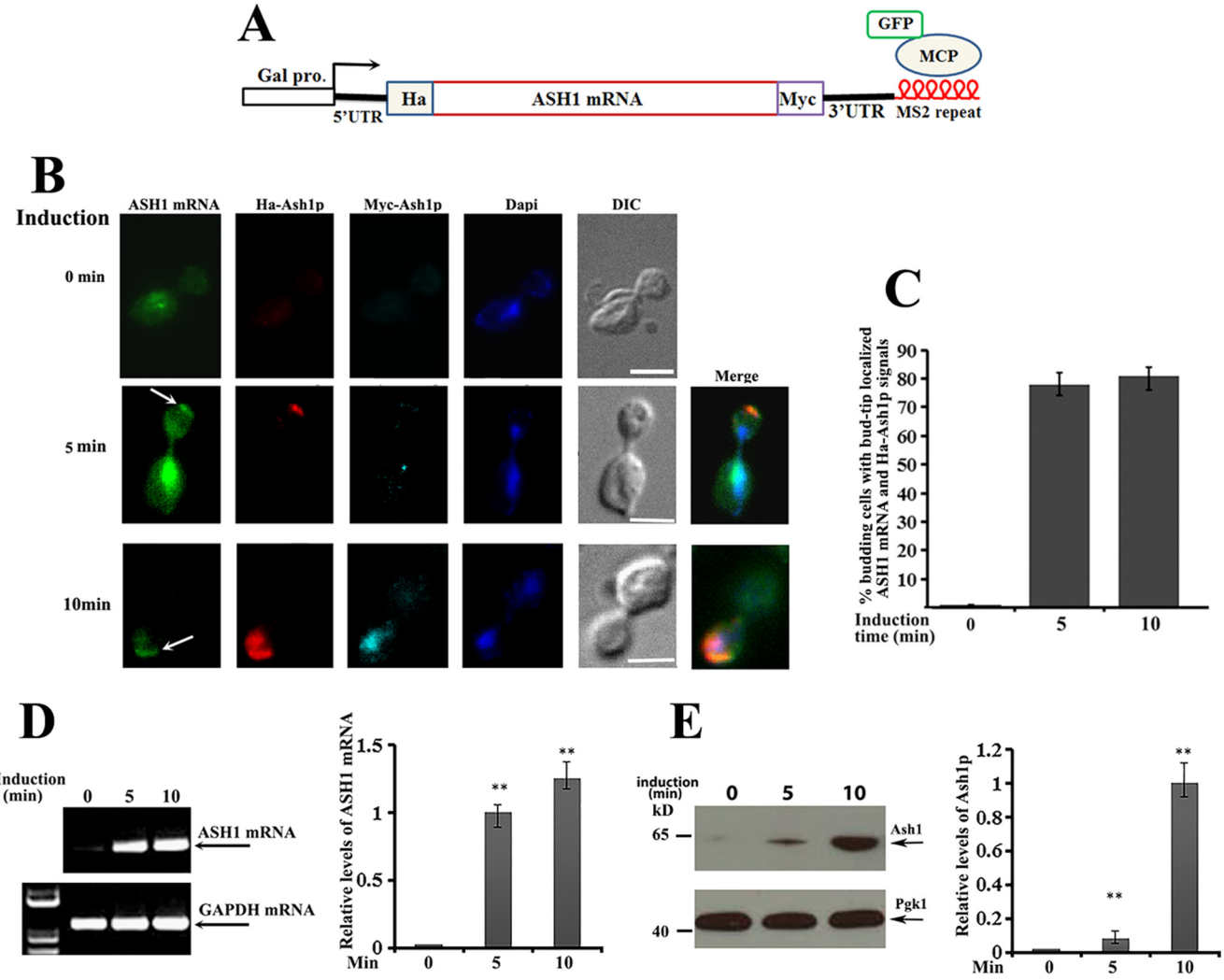


Figure 1. Translation of *ASH1* mRNA occurs after bud-tip localization. *A*, schematic presentation of the centromeric construct used to visualize *ASH1* mRNA localization and translation in *ash1* cells. The *ASH1* gene was placed under the control of a galactose-inducible promoter (*Gal1*). HA and Myc tags were separately fused onto the N and C termini of Ash1. Six repeats of MS2 stem loops were fused to the 3′-UTR of *ASH1* mRNA, which were recognized by GFP-MCP (MS2 RNA coat protein). *B*, yeast cells were cultured in 2% raffinose medium at 30 °C and induced with galactose (2%). After 0, 5, and 10 min of induction, cells were harvested and subjected to immunostaining assays. Localization of *ASH1* mRNA was visualized by GFP-MCP, and translation of Ash1 was detected by Cy3-labeled HA (*red*) or Cy5-labeled Myc (*cyan*) antibodies. The *arrows* indicate the bud-tip-localized *ASH1* mRNA. *Bar*, 3 μm. *C*, percentage of anaphase cells with localized *ASH1* mRNA and translated HA-Ash1 signals after galactose induction from three independent experiments. An average of 50–60 cells were counted each time. *D*, RT-PCR was used to detect the expression of *ASH1* mRNA after 0, 5, and 10 min of galactose induction. GAPDH mRNA was used as a positive control. *Right*, *error bars* indicate S.E. from three independent experiments. **, $p < 0.01$. *E*, full-length Ash1 after 0, 5, and 10 min of galactose induction was detected using anti-Myc antibodies by Western blotting, in which Pgk1 was used as a loading control. *Right*, mean values ± S.D. (*error bars*) were from three independent experiments. **, $p < 0.01$.

affects translation of the mRNA. Forcibly shifting non-polysomal *ASH1* mRNA into polysomes causes dissociation of Dhh1 with the transcript. Dhh1 is not recruited to *ASH1* mRNA co-transcriptionally but is possibly associated with the messenger RNA within the cytoplasm. Interestingly, Dhh1 did not directly interact with Puf6 but bound to the 5′-UTR of *ASH1* mRNA, and this binding repressed translation of the mRNA *in vitro*. These results suggest a novel function of Dhh1 in regulating localized *ASH1* mRNA translation after its bud-tip localization.

Results

Translation occurs after ASH1 mRNA localization to the bud tip

It has been reported previously that the average time to transcribe a yeast gene is about 25–50 s/kb (14–17), and the transport time for an *ASH1* mRNA reporter (from mother to the bud tip) is about 128 s (18). Based on these results, the process from transcription to localization of *ASH1* mRNA would be around 4–5 min. Due to difficulties in investigating the local translation of endogenous *ASH1* mRNA, we transformed two centromeric plasmids, one expressing HA-ASH1-Myc-MS2$_6$ mRNA (a 2.5-kb transcript) under Gal promoter control and another expressing a GFP-MCP (MS2-binding coat protein) fusion protein into an *ASH1* gene deletion strain. The fusion protein contains a nuclear localization signal, such that the protein unbound to ASH1 mRNA is sequestered within the nuclei (Fig. 1*A*). This system allowed us to study the coordinated localization and translation of *ASH1* mRNA in the anaphase cells because translational initiation of the protein could be imaged by immunostaining with HA antibodies, and fully translated Ash1 would be translocated into the bud nucleus and be detected by Myc antibodies. Fig. 1*B* shows representative

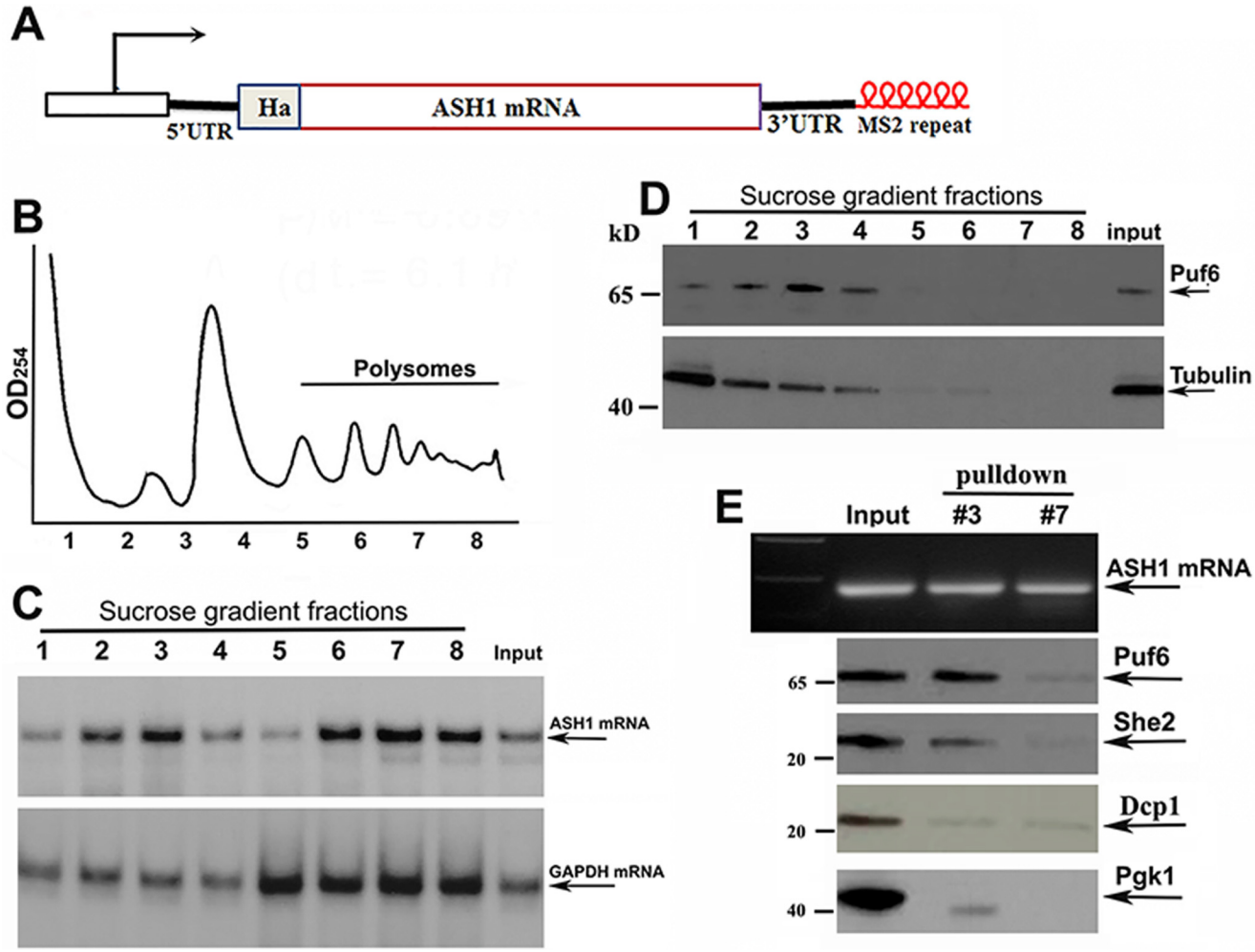


Figure 2. Puf6 associates with non-polysomal ASH1 mRNA. *A*, schematic representation of the modified endogenous *ASH1* gene. HA tag was fused to the N terminus of Ash1. Six repeats of MS2 stem loops were fused to the 3′-UTR of *ASH1* mRNA. *B*, extracts of yeast cells were fractionated in 10–50% linear sucrose gradients. An A_{254} plot corresponding to the polysomal fractions is shown. *C*, total RNAs were isolated from the input and sucrose fractions. An equal amount of RNA from each fraction was subjected to Northern blotting to detect the distribution of *ASH1* mRNA in the sucrose gradient (*arrows*) with GAPDH mRNA as an internal control. *D*, equal amounts of protein from each fraction were subjected to Western blotting for detecting the expression of Puf6. Tubulin was used as an internal control. *E*, *ASH1*-MS2 mRNA was precipitated using recombinant MBP-MCP (MBP binds to amylose resins), as shown in the *top panel*. Fractions *3* and *7* are non-polysomal and polysomal fractions. Puf6 and She2 were detected in the input and the precipitates of the non-polysomal fraction. Dcp1 and Pgk1 were used as negative controls.

images in a time course of 0, 5, and 10 min after galactose induction. GFP-labeled *ASH1* mRNA was visualized at the bud tip after 5 min of galactose induction (Fig. 1*B*, *middle panels*, *arrow*), where translational initiation could be seen by Cy3-labeled anti-HA antibody (*red*). After a 10-min induction, *ASH1* mRNA and HA-Ash1 signals were still obviously distinguished at the bud tip, whereas the fully translated Ash1, which was detected by both anti-HA and Cy5-labeled anti-Myc antibodies, was translocated into the nucleus (*cyan*) (Fig. 1*B*, *bottom panels*). Localized translation of *ASH1* mRNA after 5 and 10 min of galactose induction could be observed in about 80% of anaphase cells (Fig. 1*C*). Consistent with the imaging results, *ASH1* mRNA was identified within 5 and 10 min of induction by RT-PCR (Fig. 1*D*), and the full-length Ash1 was detected by the Myc antibody after 10 min of induction (Fig. 1*E*). Statistical analyses of the relative levels of *ASH1* mRNA and Ash1 are shown in the *right panels* of Fig. 1, *D* and *E*. These data suggest that translational initiation of *ASH1* mRNA could occur directly after localization.

A portion of ASH1 mRNAs could be translationally repressed after localization

To examine whether all *ASH1* mRNA was translated after localization, we performed sucrose-gradient fractionation of cells expressing an endogenous HA-tagged ASH1-$MS2_6$ transcript and a His-tagged Puf6 (Fig. 2*A*). An A_{254} plot corresponding to the polysomal fractions is shown in Fig. 2*B*. Total RNAs were isolated from the sucrose fractions and were used to analyze the distribution of *ASH1* mRNA by Northern blotting (Fig. 2*C*). In comparison with GAPDH mRNA that was mostly polysomal, a substantial amount of *ASH1* mRNA was detected in non-polysomal fractions (fractions 2 and 3) as well as in the polysomal fractions (fractions 6–8). Disrupting the polysomes with EDTA shifted *ASH1* mRNA into non-polysomal fractions (supplemental Fig. S1). Because binding of Puf6 repressed *ASH1* mRNA translation during transport (12) and release of the protein by phosphorylation resulted in translational activation of the mRNA (13), we analyzed the distribution of Puf6 in

the sucrose fractions. Western blotting demonstrated that Puf6 was present in the non-polysomal fractions (Fig. 2*D*, fractions 2 and 3 of the *top panel*). We then used recombinant chimeric MBP-MCP (supplemental Fig. S2*A*; MBP binds to amylose resin) to precipitate *ASH1*-MS2$_6$ mRNA in selected sucrose fractions (Fig. 2*E*, *top*). Puf6 co-precipitated with *ASH1* mRNA in the non-polysomal fraction 3 but not in the polysomal fraction 7. She2, an essential protein of the *ASH1* mRNA localization machinery, was also detected in the precipitates of the non-polysomal fraction. An mRNA-binding and -decapping protein, Dcp1 and an abundant Pgk1 (phosphoglycerate kinase 1) were not present in the precipitates of either fraction 3 or fraction 7 (Fig. 2*E*, *bottom*). These data suggest that the non-polysomal fractions contained localized and translationally repressed *ASH1* mRNA.

Dhh1 binds to bud-tip-localized and translationally inactive ASH1 mRNA

In yeast, Dhh1 is an abundant DEAD-box helicase, implicated in translational repression and enhancement of mRNA decay (19–21). The role of Dhh1 in storing and maintaining untranslated mRNAs has been well reported (22). To test the possibility that Dhh1 or other P-body proteins could be involved in the regulation of *ASH1* mRNA translation, we prepared extracts from WT and *puf6* strains expressing an endogenous HA-tagged ASH1-MS2$_6$ transcript. We then used the recombinant MBP-MCP to pull down the *ASH1*-MS2$_6$ mRNA. RT-PCR analysis indicated that *ASH1* mRNA was successfully precipitated in both wild-type and *puf6* cells (Fig. 3*A*). The specificity of the experiment was tested by a control strain in which the MS2 stem loops were not fused to *ASH1* mRNA (Fig. 3*A*, *NC*). Analyzing the proteins co-precipitated with the *ASH1* mRNA, we found that in addition to Puf6, Dhh1 also existed in the precipitates of both WT and *puf6* cells (Fig. 3*B*), suggesting that the *in vivo* binding of the Dhh1 with *ASH1* mRNA could be Puf6-independent. Another P-bodies component, a decapping enzyme, Dcp1, was not detected in the precipitates. The interaction of Dhh1 with *ASH1* mRNA was confirmed by a reciprocal approach, in which untagged endogenous *ASH1* mRNA was detected in the precipitates of Dhh1 antibodies (Fig. 3*C*). We next precipitated *ASH1* mRNA from sucrose fractions 3 and 7 (Fig. 2*A*) and found that Dhh1 was associated with *ASH1* mRNA specifically in the non-polysomal fraction (Fig. 3*D*). Dcp1 was not detected in the precipitates (not shown). Interestingly, further experiments revealed that although Dhh1 and She2 were both identified to associate with *ASH1* mRNA, precipitation of Dhh1-bound *ASH1* mRNA in the non-polysomal fraction detected little She2 (Fig. 3*E*). This indicates that Dhh1 could bind to localized and translationally inactive *ASH1* mRNA independent of She2. Using fluorescent *in situ* hybridization (FISH) assays for *ASH1* mRNA in a yeast strain expressing Dhh1-GFP or Dcp1-GFP fusions, we found that *ASH1* mRNA was co-localized with Dhh1 in >30% of the budding cells (Fig. 3, *F* and *G*).

The roles of Dhh1 in bud-tip localization, translation, and stability of ASH1 mRNA

We have shown previously that binding of Puf6 to the PUF consensus sequences in the 3′-UTR of *ASH1* mRNA regulated localization and translation of the mRNA (12). Here, we analyzed how the Dhh1-*ASH1* mRNA interactions affect the localization and translation of ASH1 mRNA. Whereas >70% of the wild-type budding cells showed localized *ASH1* mRNA at the bud tip (red), a diminution of bud-tip localization of *ASH1* mRNA (~40%) was observed in *dhh1* cells, where about 42% of *ASH1* mRNA was diffuse in the bud (Fig. 4*A*, *bottom left*). Compared with the phenotype observed in *she2* or *puf6* cells, where the asymmetric distribution of *ASH1* mRNA was completely disrupted (12, 23), deletion of Dhh1 only resulted in delocalizing *ASH1* mRNA in the bud.

To examine the potential effect of Dhh1 in *ASH1* mRNA translation and stability, we examined the intracellular levels of Ash1 and *ASH1* mRNA in the wild-type, *dhh1*, and *dhh1-puf6* strains. The levels of Ash1 were clearly increased in the *dhh1* (~1.38-fold) and *dhh1-puf6* cells (~1.5-fold), in contrast to the wild-type strain (Fig. 4*B*). The *ASH1* mRNA levels were also relatively higher in the *dhh1* (~1.2-fold) and *dhh1-puf6* strains (~1.3-fold) when normalized to GAPDH mRNA (Fig. 4*C*). To analyze whether the increased levels of *ASH1* mRNA resulted from *DHH1* deletion, we transformed a plasmid expressing *ASH1* mRNA under the Gal promoter control into the *ash1* and *ash1-dhh1* strains. Cells were grown in medium with 2% raffinose to ~0.6 at A_{600} and were induced with 2% galactose for 10 min. The Gal promoter was repressed by the addition of glucose, and the *ASH1* mRNA levels were quantitated using RT-qPCR. The steady-state levels of *ASH1* mRNA were not obviously different between the two cell strains. In the *dhh1* cells, levels of *ASH1* mRNA were decreased by 35% at 5 min and by 62% at 10 min compared with reduction of 46% at 5 min and 70% at 10 min in the control cells (Fig. 4*D*). To further determine the role of Dhh1 in *ASH1* mRNA translation, we transformed a centromeric plasmid expressing Dhh1 protein into *dhh1* cells. We found that when Dhh1 was overexpressed, expression of Ash1 was considerably decreased, whereas the levels of *ASH1* mRNA were still mildly reduced (Fig. 4, *E*–*G*). These results indicate that Dhh1 affects both the levels of *ASH1* mRNA and protein. It seems that the impact of Dhh1 on protein levels can at least partially be explained by effects that are distinct from the effects on mRNA levels. One possibility, consistent with previous studies on Dhh1 as a translation repressor (19), is that it can act to repress translation of the *ASH1* mRNA.

Translational initiation of ASH1 mRNA is accompanied with the dissociation of Dhh1 from the transcript

We hypothesized that binding of Dhh1 to the non-polysomal *ASH1* mRNA could lead to regulation of its localized translation. To address this, we treated yeast cells with low levels of cycloheximide (CHX), which inhibited translational elongation but not initiation (24), to force the shift of ribosome equilibrium toward the polysomes (25). Using the HA-ASH1-Myc-MS2$_6$ reporter, we showed that in CHX-untreated growing cells, matured Ash1 (*red*) was synthesized and translocated into the bud nucleus, and *ASH1* mRNA was tightly localized at the bud tip (*top panels* of Fig. 5, *A* and *C*, respectively). However, in CHX-treated cells, strong HA signal was mostly seen in the bud cytoplasm, whereas very little matured Ash1 was seen in the nucleus (Fig. 5*A*, *bottom panels*). Repression of Ash1 elongation was confirmed by Western blotting using Myc antibodies,

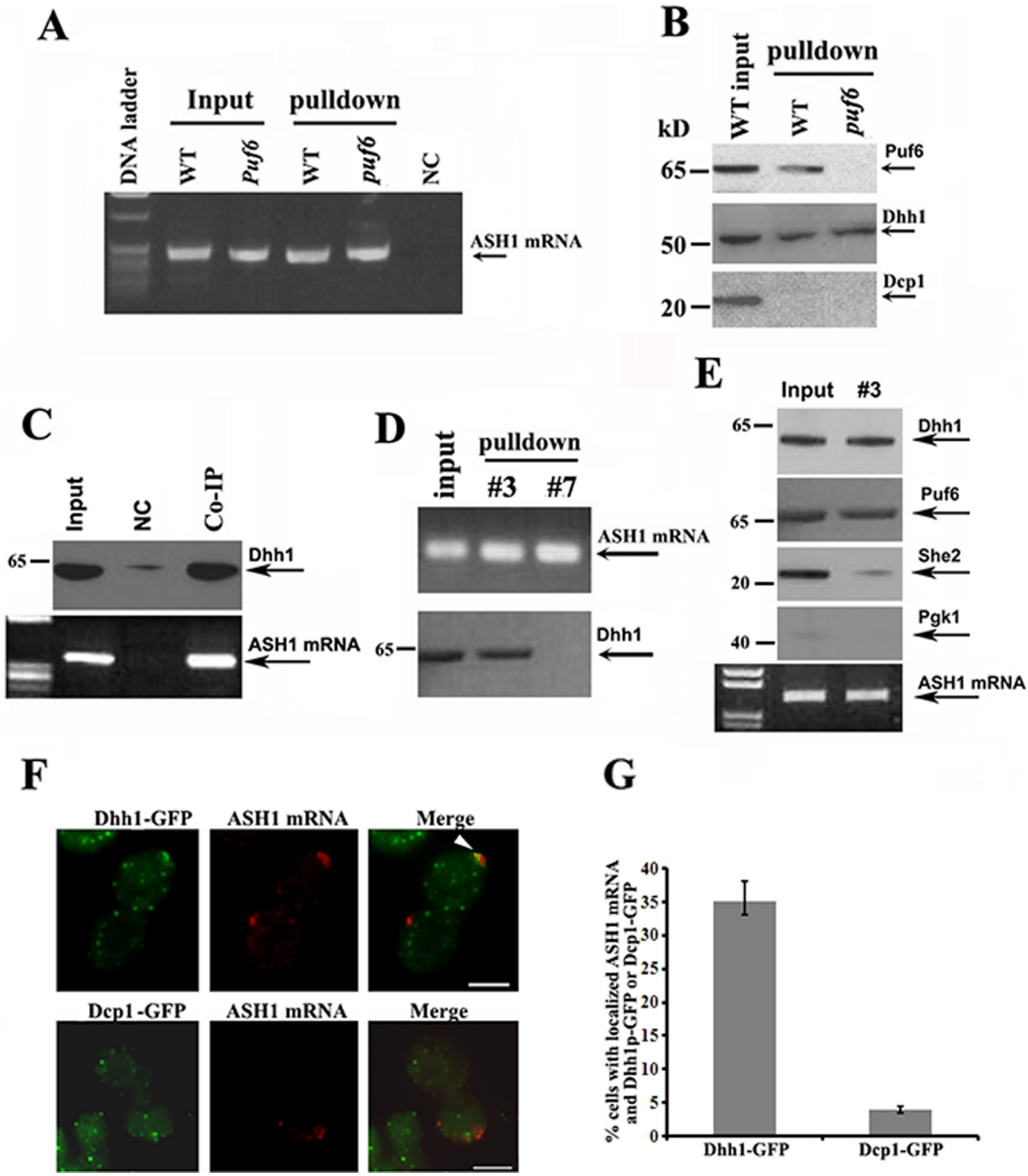
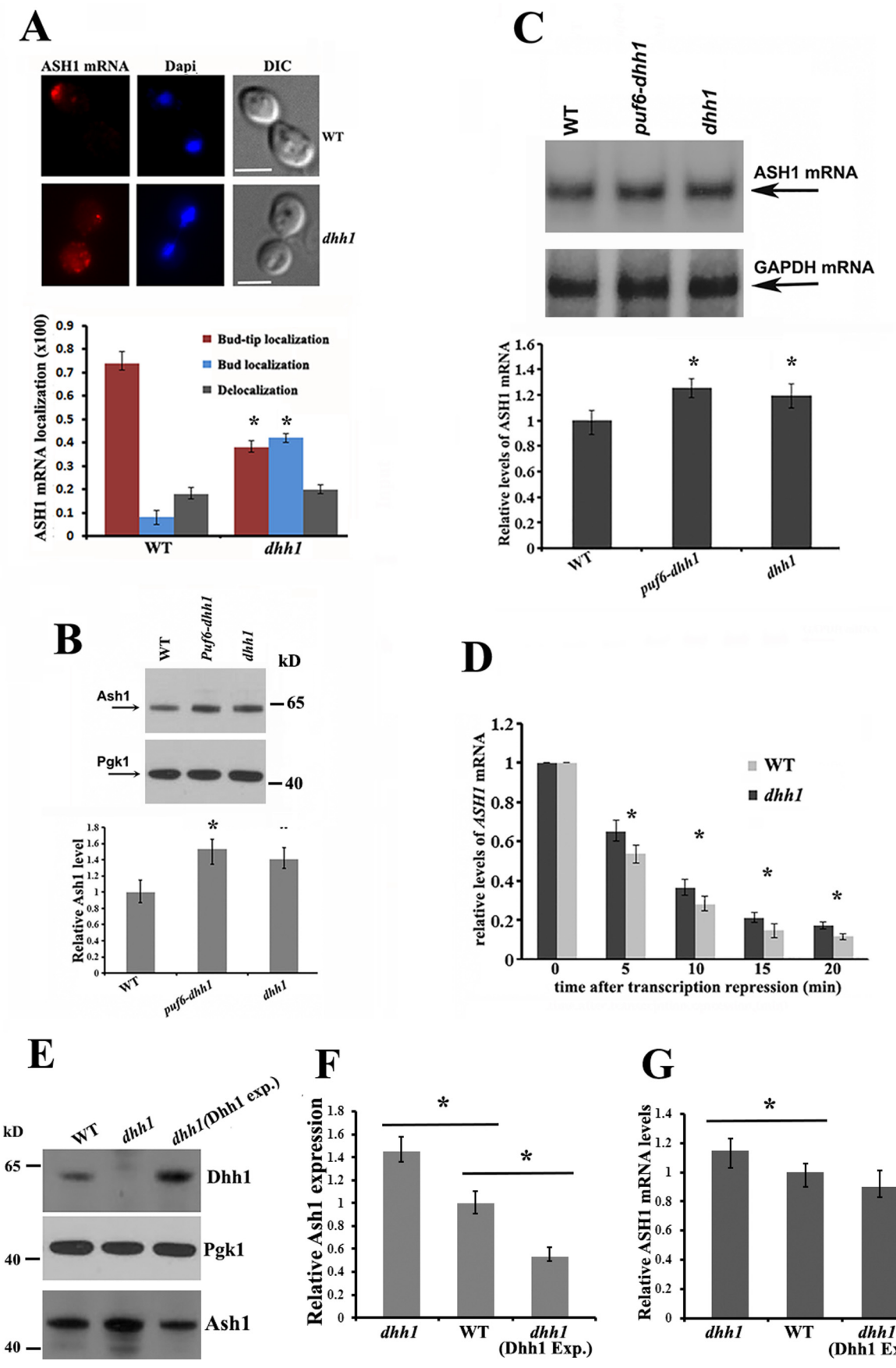


Figure 3. Dhh1 binds to localized and translationally inactive *ASH1* mRNA. *A*, *ASH1* mRNA was precipitated from extracts of WT and *puf6* cells by MBP-MCP. The precipitates were analyzed by RT-PCR. *Arrow*, amplified *ASH1* cDNA. *NC*, negative control in which the $MS2_6$ motif was not fused into *ASH1* mRNA. *B*, the potential interaction of *ASH1* mRNA with Puf6, Dhh1, or Dcp1 was analyzed by immunoblotting with the precipitates from *A*. *C*, co-IP experiments were used to analyze the association of endogenous *ASH1* mRNA with Dhh1 using Protein A beads conjugated with Dhh1 antibodies in wild-type cells. The precipitates were subjected to Western blotting and PT-PCR analyses. *NC*, negative control using Protein A beads unconjugated with the antibodies. All Western blotting experiments were carried out at least two times. *D*, *ASH1* mRNA was precipitated with MBP-MCP from total cell extracts (input) and the sucrose fractions 3 and 7 (Fig. 2*B*). Dhh1 was associated with *ASH1 mRNA* in the input and non-polysomal fraction. *E*, co-IP experiments were performed to analyze the binding status of *ASH1* mRNA with Dhh1 and She2 in cell extracts (*Input*) and non-polysomal fraction (*#3*) using Protein A beads conjugated with Dhh1 antibodies. The precipitates were subjected to Western blotting and PT-PCR analyses. *F*, FISH was performed to detect the localization of *ASH1* mRNA in yeast strains expressing Dhh1-GFP or Dcp1-GFP. The *arrowhead* in the representative images indicates colocalization of *ASH1* mRNA with Dhh1-GFP. *Bar*, 3 μm. *G*, the percentage of *ASH1* mRNA colocalizing with Dhh1 or Dcp1 was shown from three independent experiments. On average, 40–50 cells were counted in each experiment. *Error bars*, S.D.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WT
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ASH1 mRNA localization (x100)
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ASH1 mRNA
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Relative levels of ASH1 mRNA
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E
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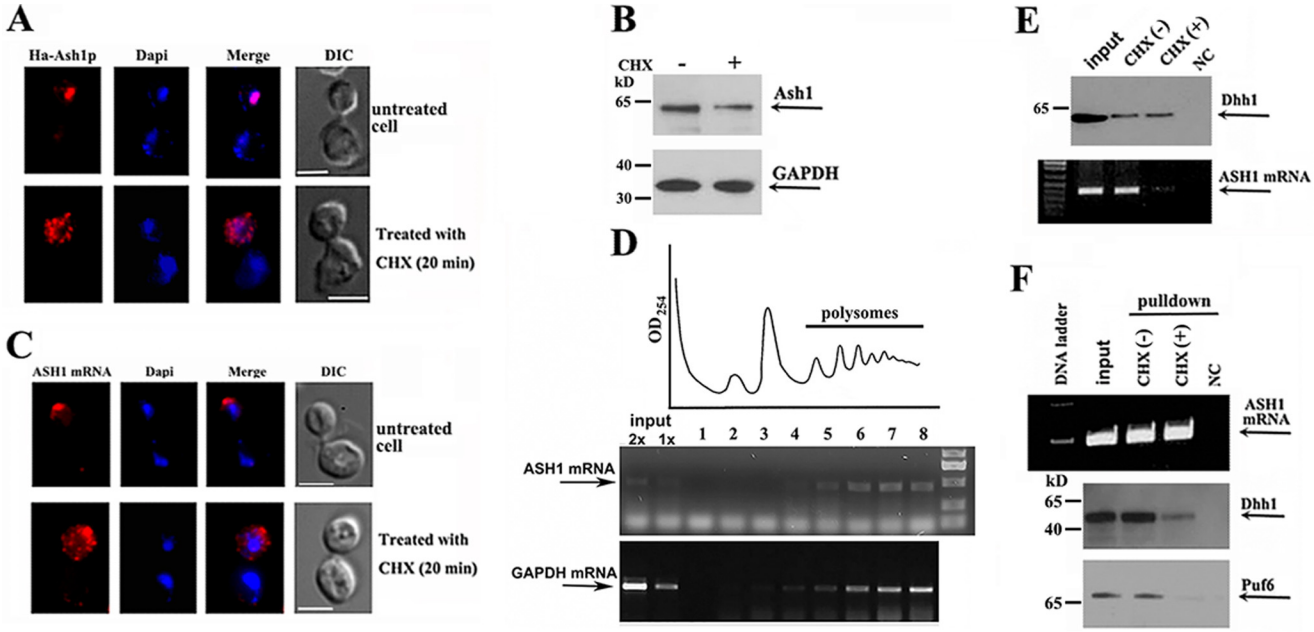

Figure 5. Activation of translational initiation results in disassociation of Dhh1 with ASH1 mRNA. *A*, fluorescent experiments were performed in the cells without or with CHX treatment. The *top panels* show a representative normal cell, in which Ash1 was fully translated and translocated into the cell nucleus, whereas in a CHX-treated cell, detected Ash1 was mostly cytoplasmic, due to the inhibition of protein elongation (*bottom panels*). *Bar*, 3 μm. *B*, Western blots showing the expression of Ash1 and GAPDH in normal and CHX-treated cells. *C*, FISH analyses showed *ASH1* mRNA that was localized at the bud tip in a normal growing cell (*top panels*) but was diffuse in the bud in a CHX-treated cell (*bottom panels*). *Bar*, 3 μm. *D*, extracts of yeast cells were prepared after treatment with cycloheximide for 20 min and were fractionated in 10–50% linear sucrose gradients. An A_{254} plot corresponding to the polysomal fractions is shown. Total RNAs were isolated from the input and sucrose fractions. An equal amount of RNA from each fraction was subjected to RT-PCR to detect the distribution of ASH1 mRNA and GAPDH mRNA in the sucrose gradient (*arrows*). *E*, immunoprecipitation assays to analyze the association of Dhh1 with *ASH1* mRNA in the cells treated with CHX. *ASH1* mRNA was co-precipitated with Dhh1 in normal growing cells but little in CHX-treated cells (*right*). *NC*, a negative control in which no antibody was used. *F*, *ASH1* mRNA was pulled down by MBP-MCP (*top*). Western blotting indicated that Dhh1 and Puf6 were co-precipitated in normal growing cells but not in CHX-treated cells (*bottom panels*). *NC*, a negative control using empty beads. The results are representative of three independent experiments.

which indicated that cells treated with CHX expressed less full-length Ash1 (Fig. 5*B*). Previous studies have reported that translation is required for proper localization of *ASH1* mRNA. Either prevention or inhibition of *ASH1* mRNA translation delocalized the mRNA in the bud (10, 26). As a result of CHX treatment, we also found that *ASH1* mRNA (*red*) was diffusely localized in the cytoplasm of the bud (Fig. 5*C*, *bottom*), probably resulting from the mRNA that was trapped in polysomes with the nascent Ash1 peptide. Sucrose gradient assays showed that *ASH1* mRNA, like GAPDH mRNA, was mostly distributed in the polysomal fractions in CHX-treated cells (Fig. 5*D*). In addition, Dhh1 and Puf6 GAPDH levels were modestly decreased in CHX-treated cells (supplemental Fig. S3, *A* and *B*). To determine the effect of the cellular association of Dhh1 with *ASH1* mRNA after CHX treatment, we immunoprecipitated Dhh1 (Fig. 5*E*, *top*) and tested for the presence of *ASH1* mRNA by RT-PCR. *ASH1* mRNA was shown in the precipitates of the untreated growing cells but was greatly decreased in that of CHX-treated cells (Fig. 5*E*, *bottom*). We alternatively pulled down *ASH1* mRNA by MBP-MCP (Fig. 5*F*, *top*) and analyzed the presence of Dhh1 and Puf6 in the precipitates. Western blotting (Fig. 5*F*, *bottom panels*) and quantitative analysis (supplemental Fig. S3*C*) indicated significantly decreased amounts of Puf6 and Dhh1 associated with *ASH1* transcript in cells treated with CHX, suggesting that the shift of non-polysomal *ASH1* mRNA into polysomes was accompanied by the dissociation of Dhh1 and Puf6.

Involvement of Dhh1 in translational regulation of other bud-localized mRNAs

Reports have shown that a number of bud-localized transcripts, including MID2 (YLR332w) and SRL1 (YOR247w) mRNAs, were associated with Puf6 post-transcriptionally (1).

Figure 4. Effects of Dhh1 in ASH1 mRNA localization, translation, and stability. *A*, FISH to determine *ASH1* mRNA localization in wild-type and *dhh1* cells. Representative images show *ASH1* mRNA localization in a wild-type budding cell and in the bud of a *dhh1* cell (*top panels*). *Bar*, 3 μm. *Bottom*, cells showing different patterns of *ASH1* mRNA localization were classified as follows: anchored (tightly localized *ASH1* mRNA at the distal bud tip); delocalized in the bud (delocalized *ASH1* mRNA confined to the bud); or delocalized in mother and bud (*ASH1* mRNA in both mother cell and bud). The percentage of localized *ASH1* mRNA in wild-type and *dhh1* cells was shown from three independent experiments. An average of 50–80 cells was counted each time. *, $p < 0.05$. *B*, Western blotting of Ash1 and Pgk1 in WT, *dhh1*, and *dhh1-puf6* cells. The *arrows* indicate the detected proteins. Relative levels of Ash1 expression were normalized to Pgk1. Mean values ± S.D. (*error bars*) from three independent experiments are shown. *, $p < 0.05$. *C*, Northern blotting analysis of *ASH1* mRNA levels in WT, *dhh1*, and *dhh1-puf6* cells. *ASH1* mRNA levels were normalized to GAPDH mRNA. Mean values ± S.D. were from three independent experiments. *, $p < 0.05$. *D*, cells expressing *ASH1* mRNA under the Gal promoter control were grown in synthetic medium with 2% raffinose to A_{600} ~0.8, followed by the addition of galactose to 2% to induce *ASH1* mRNA expression. 30 ml of culture was collected as t_0. Glucose was added to a final concentration of 3% to stop *ASH1* transcription. Cells were collected at 5, 10, 15, and 20 min. *ASH1* mRNA levels were measured by qPCR and normalized to GAPDH mRNA for each sample. *Bars*, S.E. from three independent experiments. *, $p < 0.05$. *E*, a centromeric plasmid expressing Dhh1 was transformed into *dhh1* cells. Expression of Dhh1, Pgk1, and Ash1 proteins in WT, *dhh1*, and *dhh1*(Dhh1 exp.) strains were detected by Western blotting. *F*, Ash1 expression in WT, *dhh1*, and *dhh1*(Dhh1 exp.) cells. The relative levels of Ash1 were normalized to Pgk1. Mean values ± S.D. from three independent experiments were shown. *, $p < 0.05$. *C*, RT-qPCR to analyze the levels of *ASH1* mRNA in WT, *dhh1*, and *dhh1*(Dhh1 exp.) cells. *ASH1* mRNA levels were normalized to GAPDH mRNA. Mean values ± S.D. were from three independent experiments. *, $p < 0.05$.

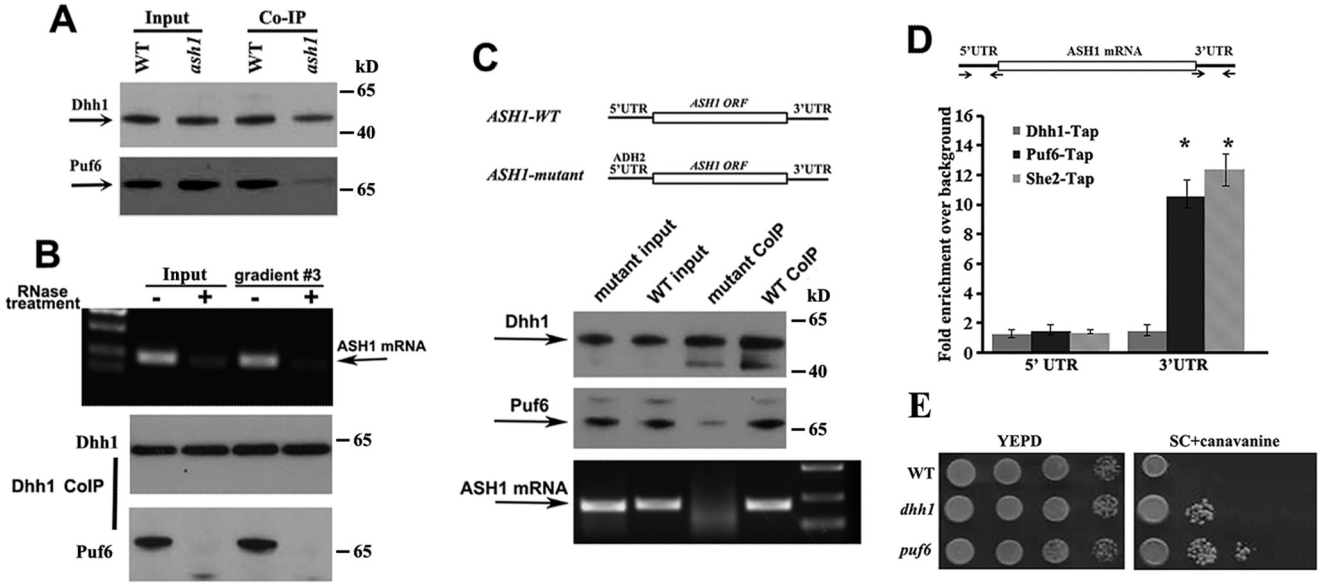

Figure 6. Dhh1 does not directly interact with Puf6; it could bind to the 5′-UTR of ASH1 mRNA. *A*, co-IP experiments using Protein A beads conjugated with Dhh1 antibodies were performed in the extracts of WT and *ash1* cells. Western blotting indicated that co-precipitation of Dhh1 with Puf6 required *ASH1* mRNA. *B*, co-IP experiments were performed in the yeast extracts and the non-polysomal fraction 3 treated with or without RNase A and RNase One. Dhh1 did not co-precipitate Puf6 when total mRNA in the extracts was degraded. *C*, *top panels*, schematic presentation of yeast strains expressing *ASH1-WT* (*5′UTR-ASH1–3′UTR*) and *ASH1-mutant* (*ADH2–5′ UTR-ASH1-3′UTR*) mRNAs. The 5′-UTR of *ASH1* is a 150-nucleotide sequence upstream of the translational initiation site of the mRNA. The 3′-UTR of *ASH1* is a 160-nucleotide sequence containing binding sites for Puf6. The 5′-UTR of *ADH2* is a 150-nucleotide sequence upstream of the translational initiation site of the mRNA. *Bottom panels*, Dhh1 was immunoprecipitated in the extracts of *ASH1-WT* and *ASH1-mutant* cells. Puf6 and *ASH1* mRNA were detected in the precipitates of WT extracts. A small amount of Puf6 and no ASH1 mRNA were detected in the precipitates of *ASH1-mutant* extracts. *Arrows*, positions of Dhh1, Puf6, and *ASH1* mRNA. The results are representative of three independent experiments. *D*, *top*, schematic presentation of the *ASH1* gene. The 3′-UTR contains the E3 localization element for Puf6 and She2 binding. *Arrows*, primers used for qPCR analysis. Amplicon lengths for 5′-UTR and 3′-UTR are 150 and 160 bp, respectively. *Bottom*, TAP-tagged Puf6, She2, and Dhh1 were used for CHIP assays. After immunoprecipitation, amplicons corresponding to 5′-UTR and 3′-UTR were amplified and analyzed. *E*, effect of *DHH1* deletion on repression of the HO promoter. 10-Fold serial dilutions of exponentially growing wild-type, *dhh1*, or *puf6* cells were spotted on YPD or SC medium containing 0.03% canavanine and incubated for 2 and 5 days at 30 °C, respectively. *Error bars*, S.E.

Interestingly, these two mRNAs were also shown to complex with Dhh1 *in vivo* (27). To determine whether blocking of translational elongation of mRNAs would also result in the dissociation of Puf6 and Dhh1 with the two transcripts, we treated a TAP-tagged Puf6 strain (12). After treatment with CHX, we immunoprecipitated Puf6 and analyzed the precipitates by Western blotting and RT-PCR. The results indicated that the two transcripts (MID2 and SLR1) as well as Dhh1 co-precipitated with Puf6 in normal growing cells. However, in CHX-treated cells, the binding capability of Puf6 to the transcripts and Dhh1 was dramatically reduced (supplemental Fig. S4*A*). This suggests that in addition to *ASH1* mRNA, Dhh1 could also be involved in the translation of other bud-localized mRNAs.

Dhh1 does not directly interact with Puf6 but binds to the 5′-UTR of ASH1 mRNA

Because Puf6 binds to the 3′-UTR of the *ASH1* mRNA (12) and Dhh1 co-precipitated with Puf6 and *ASH1* mRNA (Fig. 3), we tested whether the two proteins could interact with each other. The results show that although Dhh1 antibodies could pull down Puf6 in WT strain, it co-precipitated much less Puf6 in *ash1* cells (Fig. 6*A*). The small amount of coprecipitated Puf6 could indicate the interaction of Dhh1 and Puf6 with other mRNAs, such as MID2 and SRL1. Furthermore, when yeast extracts and the non-polysomal fraction 3 (Fig. 2) were treated with RNase A and RNase One, Dhh1 was not able to co-precipitate with Puf6 (Fig. 6*B*, *bottom panels*), indicating that association of the two proteins was mediated by *ASH1* or perhaps the other mRNAs. We then prepared yeast strains expressing *ASH1* WT mRNA (*5′UTR-ASH1ORF-3′UTR*) and *ASH1* mutant (*ADH2-5′UTR-ASH1ORF-3′UTR*), in which the 5′-UTR of *ASH1* mRNA was replaced by the 5′-UTR of *ADH2* mRNA (Fig. 6*C*, *top panels*), and performed co-IP assays using Dhh1 antibodies. *ASH1* mRNA was co-precipitated with Dhh1 in the extracts of WT cells but not in the mutant cells when the 5′-UTR of the *ASH1* mRNA was absent (Fig. 6*C*, *bottom panels*). Precipitation of Puf6 was also greatly decreased in *ASH1* mRNA mutant cells (Fig. 6*C*, *middle panels*). Because Puf6 binds to the 3′-UTR of *ASH1* mRNA (12), the result suggested that Dhh1 could bind to the 5′-UTR of *ASH1* mRNA. To further address this, we fused $MS2_6$ repeats into the 3′-ends of *5′UTR-ASH1ORF-3′UTR* (WT) and *ADH2-5′UTR-ASH1ORF-3′UTR* (5′mut) constructs and used recombinant chimeric MBP-MCP to precipitate *ASH1*-$MS2_6$ mRNA in the cell extracts. Puf6 was co-precipitated with both WT and mutant *ASH1* mRNA, whereas Dhh1 was not present in the precipitates of the mutant *ASH1* mRNA (supplemental Fig. S4*B*). These results confirmed that Dhh1 bound to the transcript through the 5′-UTR and that the association of Dhh1 with Puf6 required both 5′-UTR and 3′-UTR of *ASH1* mRNA.

Dhh1 is not recruited on ASH1 mRNA co-transcriptionally

Puf6 is co-transcriptionally recruited onto nascent *ASH1* mRNA (1). To test whether Dhh1 could also be recruited onto *ASH1* mRNA during transcription, ChIP assays were per-

formed using strains expressing endogenous C-terminal TAP-tagged Dhh1, She2, or Puf6 (supplemental Fig. S5*A*). In these assays, chromatin that was associated with She2, Puf6, or Dhh1 was immunopurified by using IgG-Sepharose, and the enriched specific regions of the *ASH1* gene were measured by qPCR. Two specific amplicons from the *ASH1* gene were analyzed; one was 5′-UTR, and the other was 3′-UTR, which contained the She2- and Puf6-binding motifs and was used as an internal control (Fig. 6*D*). As shown in the figure, control amplicons of the 3′-UTR were specifically enriched after Puf6 and She2 CHIPs, whereas no enrichment of the 3′-UTR was identified in Dhh1 CHIP. There was also no enrichment for the 5′-UTR amplicon in all ChIP assays. In contrast, *ASH1* mRNA was successfully co-precipitated with Dhh1-TAP from the cell extracts (supplemental Fig. S5*B*). These results suggest that Dhh1 was not co-transcriptionally recruited on the 5′-UTR of the *ASH1* gene.

Loss of Dhh1 function affects the HO promoter activity

To examine the physiological relevance of the *DHH1* gene to the cell's ability to control mating-type switch, we tested the influence of Dhh1 on the regulation of the HO promoter. *DHH1* was deleted in a strain in which the endogenous *CAN1* gene was under the control of the HO promoter (12, 28). The cells were sensitive to canavanine when *CAN1* was expressed, whereas inhibition of the *CAN1* gene led to tolerance to the drug. We observed that deletion of *DHH1* decreased the sensitivity to canavanine (Fig. 6*E*), although to a lesser extent than the *puf6* cells that have been known to more intensely interfere with the localization of *ASH1* mRNA. This indicates a role for Dhh1 in regulation of HO expression and, therefore, mating-type switch.

Binding of Dhh1 to the 5′-UTR of ASH1 mRNA represses its translation in vitro

We used *in vitro* RNA mobility-shift experiments to further identify the binding ability of Dhh1 to the ^{32}P-labeled 5′-UTR of *ASH1* mRNA. An RNA-protein complex was formed when the RNA probe was incubated with the extracts of WT, *puf6*, and *dcp1* cells. However, the complex formation was significantly attenuated upon incubation with the extracts of *dhh1* cells (Fig. 7*A*; complex is indicated by the *arrow*), indicating that the formation of the complex relied on Dhh1. We next expressed His-tagged Dhh1 in *Escherichia coli* and used the purified Dhh1 (supplemental Fig. S2*B*) to perform binding assays. Incubation of ^{32}P-labeled 5′-UTR of *ASH1* mRNA with recombinant Dhh1 formed a distinct RNA-protein complex, which was effectively competed by excess amounts of the unlabeled probe (Fig. 7*B*). The complex formation was specific because a supershift band was observed when Dhh1 antibody was added into the reaction (*arrow*). Dhh1 did not interact with the 3′-UTR of *ASH1* mRNA because the 3′-UTR of *ASH1* mRNA did not show any competition for the complex formation (Fig. 7*C*). Thus, the *in vitro* experiments supported the *in vivo* binding of Dhh1 to the 5′-UTR of untranslated *ASH1* mRNA.

To evaluate whether binding of Dhh1 to the 5′-UTR of *ASH1* mRNA could affect translation, we performed *in vitro* cell-free translation assays in a rabbit reticulocyte lysate system using two R-luciferase reporters; one contained the 5′-UTR of *ASH1* mRNA (160 bp), and the other contained the 5′-UTR of *ADH2* mRNA (150 bp). A progressive reduction of translation was observed when the 5′-UTR of *ASH1* reporter was incubated with increasing amounts of recombinant Dhh1. The efficiency of *in vitro* translation was reduced by $>$ 50% when the molar ratio of the reporter RNA/Dhh1 reached 1:16, whereas no obvious reduction was observed when Dhh1 was incubated with the 5′-UTR of *ADH2* reporter (Fig. 7*D*). In addition, the translational efficiency was not changed when the 5′-UTR of *ASH1* reporter was incubated with increased amounts of recombinant Dcp1 (Fig. 7*E*). Recombinant Dhh1-repressed *in vitro* translation of the *ASH1* 5′-UTR reporter was also observed in a yeast extract prepared from *dhh1* cells (supplemental Fig. S6). The translational inhibition did not result from selective RNA degradation, because the amount of the reporter RNA in the reactions did not change with increasing Dhh1 levels (Fig. 7*F*). Thus, binding of Dhh1 to the 5′-UTR of *ASH1* mRNA conferred repression of translation.

Discussion

We have reported previously that binding of Puf6 to the 3′-UTR of *ASH1* mRNA repressed its translation during transport, and this repression could be released by CK2 phosphorylation in the N-terminal region of Puf6 when the mRNA was localized to the bud tip (12, 13). Our new observations showed that although translation occurred after localization, a fraction of *ASH1* mRNA was still associated with Puf6 and was translationally inactive. The 5′-UTR of the untranslated *ASH1* mRNA was simultaneously bound to Dhh1, which served to maintain the non-polysomal status of the mRNA and cause the mRNA to be tightly localized at the bud cortex.

Post-transcriptional control of mRNA localization and translation was achieved through the concerted action of RNA-binding factors and the translation machinery (10, 29–31). The fact that Dhh1 coordinates with Puf6 to regulate the translation of *ASH1* mRNA provides additional information for the importance of localized translation in relation to cell phenotype. The fact that activation of the non-polysomal *ASH1* mRNA was accompanied with Dhh1 dissociation strongly suggested that Dhh1 was indeed associated with translationally repressed *ASH1* mRNA, and this interaction requires the 5′-UTR of the transcript. The suppressive role of Dhh1 for Ash1 translation was also determined by the *in vivo* overexpression analysis and the *in vitro* translation assays. Binding of Dhh1 to the 5′-UTR of *ASH1* mRNA was most likely due to the involvement of the protein in the regulation of translational initiation. This could be partly supported by the finding that Ash1 expression was decreased when Dhh1 was overexpressed and that increasing translational initiation by CHX treatment resulted in the dissociation of the protein from *ASH1* mRNA. Because phosphorylation of Puf6 at the bud tip by CK2 decreased its binding ability to *ASH1* mRNA and eliminated the translational repression (13), it is possible that CK2 could also act on Dhh1 to release its binding.

ASH1 mRNA contains four localization elements for She2 binding, which make the localization of the mRNA more precise and efficient (7). Localized translation of *ASH1* mRNA

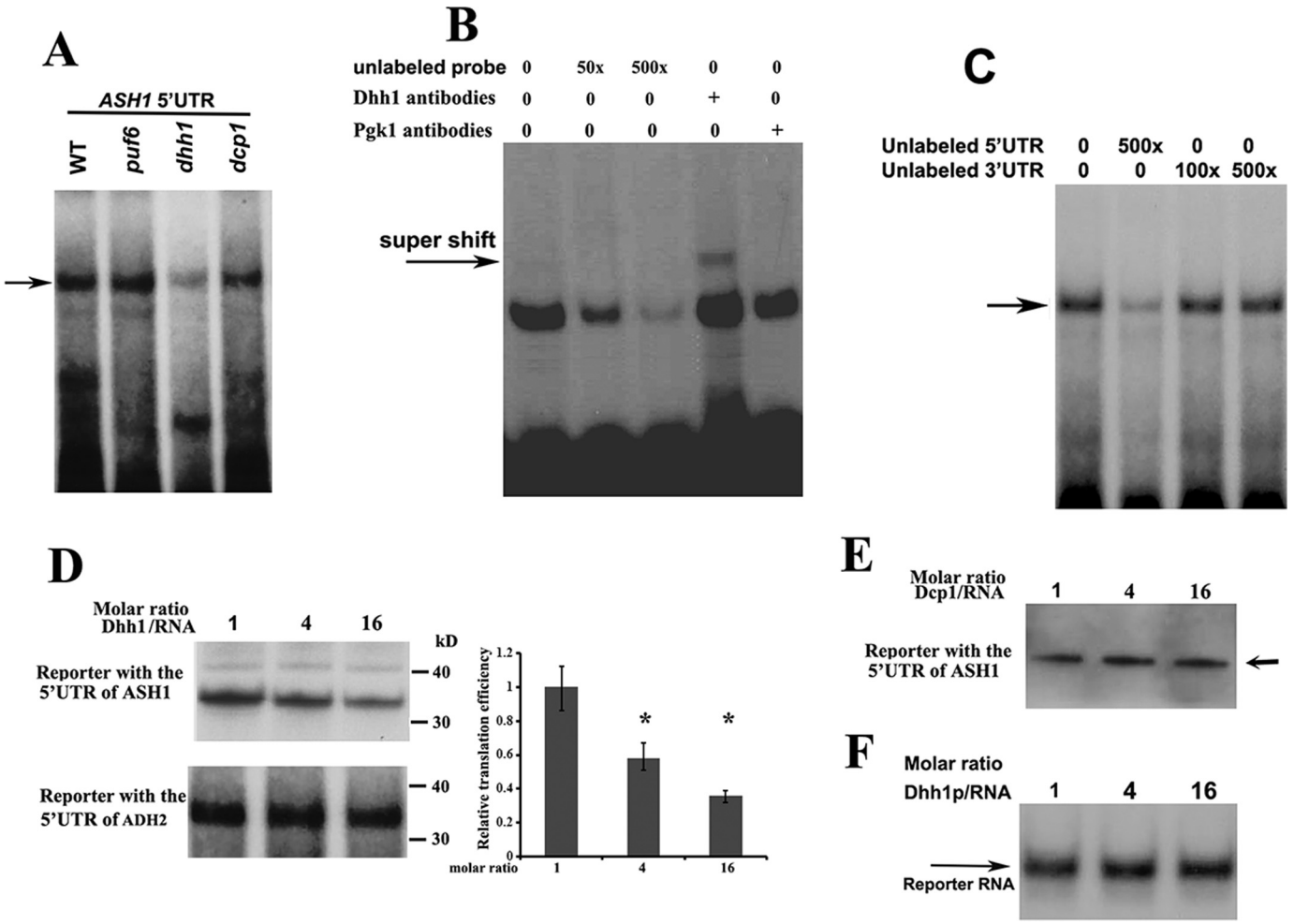

Figure 7. Dhh1 binds to the 5′-UTR of ASH1 mRNA and represses its translation *in vitro*. *A*, aliquots of ^{32}P-labeled 5′-UTR of *ASH1* mRNA were incubated with extracts prepared from WT, *puf6*, *dhh1*, and *dcp1* strains. RNA-protein complexes (*arrow*) were formed in the extracts when Dhh1 was expressed. *B*, ^{32}P-labeled 5′-UTR of *ASH1* mRNA was incubated with recombinant Dhh1 and an increased amount of unlabeled RNAs or Dhh1 antibodies. *Arrow*, a supershift of the 5′-UTR-Dhh1 antibody. *C*, binding of Dhh1 to the 5′-UTR of *ASH1* mRNA was not competed by the unlabeled 3′-UTR of *ASH1* mRNA. *D*, *left*, autoradiography of *in vitro* translated [^{35}S]methionine-labeled luciferase protein using the RNA templates containing the 5′-UTR of *ASH1* mRNA or the 5′-UTR of *ADH2* mRNA in the presence of a 1-, 4-, and 16-fold excess molar ratio of recombinant Dhh1. *Right*, relative translational efficiency of RNA template containing the 5′-UTRs of *ASH1* mRNA was shown after normalizing to the RNA templates containing the 5′-UTR of *ADH2* mRNA. Mean values ± S.D. (*error bars*) were from three independent experiments. *, $p < 0.05$. *E*, *in vitro* translation of [^{35}S]methionine-labeled luciferase protein using the RNA templates containing the 5′-UTR of ASH1 mRNA and 1-, 4-, and 16-fold excess molar ratio of recombinant Dcp1. *F*, a urea gel showing that the ^{32}P-labeled reporter RNA used for *in vitro* translation did not degrade over the course of the experiments in *D*. The experiments were carried out three times.

could also be finely controlled. This explains why Dhh1 is involved in the regulation of localized translation of *ASH1* mRNA. However, compared with *puf6* cells, in which *ASH1* mRNA was widely delocalized in both the mother and daughter cells (12), the mRNA in the *dhh1* cells was mostly diffusely localized in the buds. As a result, we observed a milder effect of Dhh1 on the regulation of the HO promoter when compared with the mutation in *puf6*. In addition, unlike Puf6 that was recruited into *ASH1* mRNA during transcription (1), Dhh1 was shown to associate with the transcript within the cytoplasm, suggesting that Dhh1 might be more involved in the regulation of *ASH1* mRNA translation after localization. We hypothesized that after bud-tip localization, some *ASH1* mRNA could be programmed for immediate translation, whereas other *ASH1* mRNA could be stored. In this case, binding of Dhh1 to translationally inactive *ASH1* mRNA could primarily result in temporal repression, storage, and then decay if no more protein synthesis is required.

In *S. cerevisiae*, Dhh1 is a DEAD-box RNA helicase and is also a major P-body component involved in translational repression and mRNA decapping (20). Recent studies indicated that Dhh1 represses mRNA translation in a way that does not rely on the translation initiation factors and that Dhh1 connects translation to mRNA decay by monitoring codon optimality (32, 33). Because Dhh1 binds to the 5′-UTR of *ASH1* mRNA, we feel that Dhh1 could be more implicated in the control of translational initiation of the mRNA. Deletion of the *DHH1* gene not only resulted in a defect in mating through the down-regulation of Ste12 expression (34) but also affected the mating-type switch due to the dysregulation of HO expression. In addition, the roles of the Dhh1 family in translational repression and enhancement of mRNA degradation have also been well studied in higher eukaryotes (19, 21). Dhh1 homologs in *Drosophila* (Me31B), *Caenorhabditis elegans* (CGH-1), and *Xenopus* (Xp54) have been implicated in translational repression and storage of maternal mRNAs (35–37).

Table 1
Yeast strains used in this study

Strain	Genotype	Source/Reference
K4452	Mata, *leu2-3, 112, ura3, his3, ade2-1, ho can1-100*	Ref. 28
K4452-*ash1*	K4452, *ash1*::*KAN*	Ref. 28
K4452-*ash1,* Ash1-Myc9	K4452-*ash1, ASH1-myc9*::*LEU*	Ref. 3
K4452-*ash1, puf6*	K4452-*ash1, puf6*::*TRP*	Ref. 12
K699	Mata, *ura3, leu2-3, 112, his3-11, trp1-1, ade2-1, ho can1-100*	Ref. 28
K699-*puf6*	K699, *puf6*::*TRP*	Ref. 12
K699-*puf6,* Puf6-His_6	K699-*puf6, Puf6-HIS$_6$*::*LEU*	This study
K4452-*ash1,* HA-ASH1-$MS2_6$	K4452-*ash1, HA-ASH-MS2$_6$*::*URA*	This study
K4452-*ash1,* HA-ASH1-Myc-$MS2_6$, MCP-GFP	K4452-*ash1, HA-ASH1-myc-MS2$_6$*::*LEU, MCP-GFP*::*TRP*	This study
K4452-*ash1,* HA-ASH1-$MS2_6$, Puf6-His_6	K4452-*ash1, HA-ASH1-myc-MS2$_6$*::*LEU, Puf6-His$_6$*::*TRP*	This study
K4452-ash1, 5′UTR-ASH1–3′UTR	K4452-*ash1*, 5′UTR-ASH1–3′UTR::LEU	This study
K4452-ash1, ADH2 5′-UTR ASH1–3′-UTR	K4452-*ash1*, ADH2 5′UTR ASH1–3′UTR::LEU	This study
K4452-*dhh1*	K4452, *dhh1*::*URA*	This study
K699-*dhh1*	K699, *dhh1*::*URA*	This study
K4452, Dhh1-GFP	K4452, *DHH1-GFP*::*TRP*	This study
K4452-*puf6, dhh1*	K4452-*puf6, dhh1*::*URA*	This study
K699-*puf6, dhh1*	K699-*puf6, dhh1*::*URA*	This study
K4452, Dhh1-His_6	K4452, *Dhh1-HIS$_6$*::*LEU*	This study
K699, Dhh1-TAP	K699, *DHH1-TAP*::*TRP*	This study
K699, Puf6-TAP	K699 PUF6-tap:TRP	Ref. 12
K699, She2-TAP	K699 SHE2-tap:TRP	Ref. 12

A recent global analysis of yeast mRNPs failed to identify consensus sequences for Dhh1 binding (27); however, an *in vitro* analysis has indicated that recombinant Dhh1 showed a strong preference for binding to adenine-rich nucleotides (38). We found that Dhh1 interacted with the 5′-UTR of *ASH1* mRNA that is not adenine-rich. Although the exact sequence element for Dhh1 binding has yet to be identified, our results indicated that interaction of Dhh1 with the 5′-UTR of *ASH1* mRNA is required for regulation of Ash1 translation. Binding of the Dhh1 to *ASH1* mRNA did not result from using the system of MS2-tagged RNA and MCP-GFP (39), because the binding was also confirmed when the mRNA was not MS2-tagged and when the MCP-GFP was not used. Our future work will be to identify the signal that determines the fate of untranslated *ASH1* mRNA: to be translated or to be stored for degradation.

In conclusion, we identified a potential regulatory mechanism for localized *ASH1* mRNA translation in which not all of the mRNA was translated after localization. Untranslated mRNA was repressed and temporally stored through the interaction with Dhh1 and Puf6. Thus, translation of localized *ASH1* mRNA could be precisely regulated by Dhh1, which bound to the 5′-UTR, and Puf6, which bound to the 3′-UTR of the mRNA. Interference of the coordination due to the lack of Dhh1 could affect *ASH1* mRNA localization and translation.

Experimental procedures

Growth medium, yeast strains, and plasmids

Strains used in this study are listed in Table 1. Yeast cells were grown in either synthetic medium lacking the nutrients indicated or rich medium. Transformation was performed according to the protocol of Gietz *et al.* (40). Unless otherwise stated, standard genetic techniques were used. Tagging, insertion, and deletion were performed by PCR-based homologous recombination using cassettes described previously (12, 41). The deletion was verified by colony-PCR analysis to confirm that the insertion and replacement were in the expected locus.

Plasmid construction

Plasmids used in this study were constructed using standard techniques. The plasmids YCP111-Gal-HA-ASH1-Myc-MS2 and YCP111-HA-ASH1-MS2 were derived from plasmid YCP111-Gal-MS2-LacZ-E3. These plasmids contain full-length 5′-UTR as well as the coding region of *ASH1* mRNA and six MS2 sites after the 3′-UTR of ASH1 mRNA for MCP binding. Plasmid YCP33-GFP-MCP was constructed previously (18). Plasmids YCP111-GFP-Dhh1 and YCP111-GFP-MCP were derived from plasmid YCP33-GFP-MCP. Plasmids YIP211-HAASH1-MS2, YIP211-5′UTR-ASH1-3′UTR, and YIP211-ADH2-5′UTR-ASH1-3′UTR were derived from the plasmid pXR193 (42). Plasmid pSP64-ASH-5′UTR was derived from plasmid pSP64-ASH1-3′UTR (12).

In situ hybridization and immunofluorescence

Yeast cells were grown in the appropriate culture medium to early or mid-log phase and were processed for FISH as described previously (12). To perform FISH, yeast spheroplasts were hybridized with a pool of Cy3-conjugated ASH1 DNA oligonucleotide probes or Cy3-conjugated MS2 probes (3, 43). Immunofluorescence was performed using a protocol described previously (42). For detecting HA-tagged or Myc-tagged Ash1 proteins, the mouse Cy3-conjugated anti-HA or Cy5-conjugated anti-Myc antibodies (Roche Applied Science) were used in a 1:200 dilution in 1× PBS and 0.1% BSA.

Preparation of yeast extracts

Yeast cells were cultured to A_{495} 1.0 and were rapidly harvested by centrifugation at 3,200 × *g* for 5 min at 4 °C. Where indicated, cycloheximide (50 μg/ml) was added to the cultures for 20 min. All subsequent steps were carried out in a cold room. After washing with 10 ml of cold buffer (50 mM Tris-Cl (pH 6.8), 100 mM NaCl, 30 mM $MgCl_2$, or 50 μg/ml), the cells were pelleted again and resuspended in 800 μl of the buffer containing 20 units/ml RNasin and 2× protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), 100 μg/ml aprotinin, 100 μg/ml pepstatin, 100 μg/ml leupeptin) in 2-ml tubes containing 800 μl of glass beads. Cells were lysed in a FastPrep (MP Biomedicals) 4 times for 60 s each time. Cell debris and beads were removed from the extract by centrifugation for 15 min at 8,000 × *g*. To prepare the extracts for *in vitro* translation

Table 2
List of primers used for qRT-PCR and qPCR

Gene	Forward primers	Reverse primers
ASH1 5′-UTR	GGCTCCTGCTCAAAAAAAGAGG	CCTCTAGAGTCGACTTTTTTTGC
ASH1 3′-UTR	TACATGGATAACTGAATCTCTTTC	GCGGCGTGTCGAATGAAAATG
ASH1 mRNA	GATCATCCTCACCTGTGCGTC	CTCTACTGTCTCACCGTTCAAG
GAPDH	GAGTCAACGGATTTGGTCGT	TGGGATTTCCATTGATGACA
DHH1	TCCTAATAGAAAGATAGACGCAG	CCGTTTCGTCATTTTCAGCCAC
DCP1	GTACATCTCTGCGCATTTTTCTC	GTAGCACGCCCTCTTCTACTAC
ADH2 5′-UTR	TCGCTACTGGCACTCTATTTATAG	CTATCAACTATTAACTATATCGT

assays, the obtained supernatant was subjected to centrifugation (4 °C, 15,000 × *g*, 30 min) again.

Sucrose-gradient fractionation

500 μl of yeast extracts were loaded onto 10-ml, 10–50% linear sucrose gradients and fractionated at 35,000 rpm for 2 h in an SW41 rotor (Beckman). Fractions (1.25 ml each) were collected from top to bottom, and the A_{254} profile was monitored.

Northern and Western blotting

For Northern blotting, total RNA was extracted, and equal amounts of total RNA were separated by agarose gel electrophoresis. RNA was transferred to a Hybond membrane and incubated with dCTP-^{32}P-labeled cDNA probes for the *ASH1* gene and the *GAPDH* gene. Hybridization and autoradiography were performed as described previously (44). For immunoblotting, equal amounts of cell extracts were resolved into a 4–12% gradient SDS-PAGE (Invitrogen), followed by electroblotting onto a nitrocellulose membrane. Western blotting was performed using one of the following primary antibodies: anti-His, anti-HA, anti-Myc, anti-Dhh1, anti-Dcp1, anti-She2, anti-GAPDH, or anti-Pgk1. After incubation with HRP-conjugated secondary antibodies, proteins were detected with an enhanced chemiluminescence detection kit (Millipore). The intensity of the protein bands was quantified using ImageJ software (NIH). Relative protein levels were determined after normalization to an internal protein control.

Expression and purification of recombinant MBP-MCP and Dhh1

Recombinant MBP-MCP protein was expressed and purified as described previously (45). A His_6-tagged cDNA encoding Dhh1 or Dcp1 protein was PCR-amplified from yeast genomic DNA and cloned into a donor plasmid pET23a plasmid (Novagen) into the NdeI and BamHI sites. The plasmid, after DNA sequence analysis, was transformed into *E. coli* (DE21) cells and expressed. The method for purifying recombinant His_6-tagged Dhh1 or Dcp1 protein was as mentioned previously (45).

In vitro pulldown experiments, RT-PCR, and Western blotting

Yeast extracts from exponentially growing cell cultures were prepared as described above. For precipitation of Dhh1-associated proteins, a total of 100 μl of extracts were incubated with 2 μg of anti-Dhh1 IgG (Santa Cruz Biotechnology, Inc.) or control IgG and 20 μl of Protein A beads (Sigma) for 4–6 h at 4 °C with gentle shaking. Where indicated, RNase A (10 μg/100 μl) and RNase One (100 units; Promega) were added. After washing extensively with DEPC-treated PBS, the beads were suspended in 40 μl of water, and the proteins were eluted by boiling for 5 min. Alternatively, His-tagged recombinant Dhh1 was attached to nickel-nitrilotriacetic acid beads for pulldown experiments. The methods for precipitation of the *ASH1* mRNA complex using MBP-MCP recombinant protein were performed as mentioned previously (45). For reverse transcription, TRIzol-extracted RNA was used as template. All PCRs were performed for 25–30 cycles using the primers specific for *ASH1* mRNA and an internal control. The intensity of the DNA bands was quantified using ImageJ software (National Institutes of Health). Relative levels of *ASH1* mRNA were normalized to the levels of the control. Primers used for RT-PCR and PCR experiments are shown in Table 2.

ChIP

Puf6-TAP and She2-TAP strains were established previously, and the construction of the C-terminal insertion cassette for tap-tagging of the *DHH1* gene was described previously (12). Expression of Dhh1-Tap, Puf6-Tap, and She2-Tap was from their corresponding endogenous loci. For each ChIP, three independent 50-ml early log phase cultures were used. To increase the number of *ASH1* transcripts, cells were synchronized for 2 h using Nocodazole at the final concentration of 15 μg/ml. IgG-Sepharose 6 Fast Flow (GE Healthcare) was used for the experiments, and the procedure for CHIP assays was as mentioned previously (1). Quantification of the immunoprecipitated DNA was performed by RT-qPCR (S1000TM thermal cycler Bio-Rad) using the SYBR Green PCR core reagent system. Each 20-μl PCR contained 10 μl of qPCR mix, 2 μl of DNA, and a 300 nM concentration of each of the primers. PCR was performed in the following conditions: activation of the reaction for 30 s at 95 °C, followed by 35 cycles of 10 s at 95 °C and then 30 s at 57 °C for the annealing and extension steps. Cycle thresholds (*Ct*) for each triplicate of samples were averaged, and ChIP enrichment was calculated by dividing the amount of ChIP DNA over control DNA using the formula, $2^{-\Delta\Delta Ct}$.

HO promoter activity assay

The effect of *DHH1* on HO expression was determined as described by Jansen *et al.* (28). Briefly, 10-fold serial dilutions of exponentially growing wild-type (K4535), *puf6*, or *dhh1* cells were spotted on YPD or SD medium contain 0.03% canavanine and incubated for 2 and 5 days at 30 °C, respectively.

Gel mobility-shift assay

A 150-bp cDNA fragment encoding 5′-UTR of *ASH1* mRNA was PCR-amplified and subcloned into pSP64 plasmid (Pro-

mega). The plasmid encoding 160 bp of the 3′-UTR of *ASH1* mRNA was constructed previously (12). ^{32}P-Labeled RNA probes were *in vitro* generated by SP6 RNA polymerase from pSP64-ASH1 5′-UTR and pSP64-ASH1 3′-UTR constructs. Transcribed RNA probes were purified after resolving in a 6% denaturing gel. RNA-protein gel-shift assays were performed at room temperature as described previously (12). The RNA-protein complexes formed were separated by electrophoresis in a 4% native gel and visualized by autoradiography. To establish the specificity of RNA-protein interactions, competition assays were performed by preincubating the cell extracts or the recombinant Dhh1 with unlabeled specific or nonspecific RNA competitors.

In vitro translation assays

In vitro translation assays were performed as described previously (12). Briefly, capped mRNA reporters with the 5′ UTR of *ASH1* mRNA or the 5′ UTR of *ADH2* mRNA were transcribed from the plasmid, pC3.1-5′UTR-Luciferase, using a T7Message Machine kit (Ambion). *In vitro* translation was performed in 25 μl of rabbit reticulocyte lysate (Promega) containing 10 nM of the mRNA templates and [^{35}S]methionine as a tracer to monitor the translated proteins in the presence of increasing amounts of recombinant Dhh1 and Dcp1. Where indicated, a yeast extract prepared from *dhh1* cells was used for the *in vitro* translation assays. Translated proteins were detected by autoradiography after separation by a 12% SDS-polyacrylamide gel. Signal intensities were determined by a PhosphorImager screen (Molecular Dynamics) and quantified using ImageQuant software.

Analysis of ASH1 mRNA and Ash1 localization

The method of quantitative measurement on the localization of *ASH1* mRNA has been described previously (42). By microscopy, yeast cells in late anaphase were scored for localized or delocalized *ASH1* mRNA in each experiment. *ASH1* mRNA was considered as bud-tip-localized when it was predominantly in the bud tip (crescent localization). *ASH1* mRNA was considered as diffuse in the bud when it was not tightly localization at the bud tip. For each experiment, ~100 budding cells were scored.

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